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(75) Inventors/Applicants *(for US only)*: **GYULEMETOVA, Roika** [BG/BG]; Persenik Str., bl. 119, et.17, app.98, 1164 Sofia (BG). **SHOPOVA, Daniela** [BG/BG]; 94a, Kostenski Vodopad Str., et.8, app.22, 104 Sofia (BG). **DIMOV, Valentin** [BG/BG]; 25, Fritiof Nansen, vh. A, 1000 Sofia (BG).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *of inventorship (Rule 4.17(iv)) for US only*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: STANDARDISED STEROID SAPONIN MIXTURE, A METHOD OF ITS OBTAINING AND APPLICATION

(57) Abstract: The invention relates to a standardised steroid saponin mixture obtained from the epigeous part of Tribulus terrestris L., containing more than 80 per cent of furostanol saponins, a method of obtainment and its application as an immunostimulator and immunomodulator.

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STANDARDISED STEROID SAPONIN MIXTURE, METHOD OF ITS OBTAINING AND APPLICATION

FIELD OF THE INVENTION

The present invention relates to a standardised steroid saponin mixture obtained from the epigeous part of *Tribulus terrestris L.* for application in the synthesis of drugs exerting the already known aphrodisiac effect in addition to drugs with new indications. The invention pertains also to the method of preparing of a standardised steroid saponin mixture obtained from the epigeous part of *Tribulus terrestris L.*

BACKGROUND OF THE INVENTION

A method of isolating a standardised steroid saponin mixture obtained from the epigeous part of *Tribulus terrestris L.* (BG Patent 52085) is know to exist, the said method consisting in subjecting the plant material to extraction using an up to 70 per cent solution of low alcohol, ethanol in particular, then chloroform treatment and extraction from the water solution using water-saturated n-butanol, followed by concentration of the butanol extract up to $1/8^{th}$ of the volume, after which the residue thus obtained is separated and dried, while the residues from the mother liquors are water-dissolved and then added directly to the chloroform-treated water solution. According to the examples described, a standardised mixture is thus obtained, the said mixture containing 50 to 55 per cent of furostanol saponins, calculated on the basis of protodioscin. Harvest is between 1.7 to 2 per cent depending on the saponin content in the plant material.

The problem that the present invention has solved is the extraction from the epigeous part of *Tribulus terrestris L.* of a substance containing a high level of furostanol saponins, which can be used to prepare new drugs with new indications.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, the problem has been solved by obtaining a standardised steroid saponin mixture containing 80 per cent of furostanol saponins. The method of preparation consists in subjecting the ground plant material to extraction using aqueous methanol solvent with a water/methanol ratio ranging from 1:1 to 1:99 at a temperature from 52 to 55°C. The extract is then treated with active carbon and after filtration is vacuum concentrated until methanol is fully removed. The aqueous concentrate is subsequently subjected to extraction with methylene chloride. Methylene chloride is then distilled off and regenerated for further use in the method. The purified aqueous solution is acidified with hydrochloric acid up to pH 4 and is subjected to extraction with water-saturated n-butanol by the known method. Butanol extracts are then washed with a sodium chloride solution and vacuum concentrated to obtain a dry residue which is dissolved in a low alcohol. Thereafter, the concentrate is either added drop-by-drop to acetone and left at a temperature of 10-15° C for about 24 hours, then filtered, washed with acetone and dried, or washed with a 0.5-1.5-per cent sodium hydroxide solution and, after separating the two layers, the butanol layer is washed with water to reach pH = 7, then the butanol extracts are vacuum concentrated until the organic solvent is fully removed and the aqueous solution is dried. A soft and light bright yellow powder containing more than 80 percent of furostanol saponins is thus obtained. Harvest from air-dried plant material is about 3 per cent.

This new product containing more than 80 per cent of furostanol saponins can be used to prepare new drugs exerting an immunomodulating and/or immunostimulating effect.

EXAMPLES

EXAMPLE 1

One tonne of ground plant material is subjected to extraction using a 70- per cent methanol aqueous solution at 50° C until exhaustion. The extract is drawn off, then active carbon is added thereto. The resultant is passed

through a filter press. The aqueous concentrate thus obtained is subjected to a triple extraction with methylene chloride in a ratio of 1:2, 1:1, and 1:1 respectively. The organic solvent is distilled of and regenerated, while cube residue is disposed of. The purified aqueous extract is acidified with hydrochloric acid up to pH 4 and subjected to an eightfold extraction with water n-butanol in a ratio of 1:1. Butanol extracts are washed with a 5-per cent sodium chloride solution and the concentrated until a dry residue is obtained under vacuum at 60° C and then dissolved into methanol. The mixture is slowly dropped into acetone, left at a temperature of 10° C for 20 hours and finally Nutch-filtered, washed with acetone and dried.

A light yellow powder containing 85 percent of furostanol saponins is obtained as a result. Harvest is 3 per cent from air-dried plant material.

The product analysis is performed on the basis of protodioscin. (R. Gyulemetova, M. Tomova, M. Simova, T. Pangarova, Pharmazie, 37, H.4 1982).

EXAMPLE 2

One tonne of ground plant material is subjected to extraction using a 70- per cent methanol aqueous solution at 50° C until exhaustion. The extract is drawn off, then active carbon is added thereto. The resultant is passed through a filter press. The aqueous concentrate thus obtained is subjected to a triple extraction with methylene chloride in a ratio of 1:2, 1:1, and 1:1 respectively. The organic solvent is distilled of and regenerated, while cube residue is disposed of. The purified aqueous extract is acidified with hydrochloric acid up to pH 4 and subjected to an eightfold extraction with water saturated n-butanol in a ratio of 1:1. Butanol extracts are washed with a 1-per cent sodium hydroxide solution, the ratio of alkaline water to butanol extract being 1:1 and after separating the two layers, the butanol layer is washed with water up to pH 7. Butanol extracts are then vacuum concentrated until the organic solvent is fully removed, after which the aqueous solution is dried.

A light yellow powder containing 85 percent of furostanol saponins is obtained as a result. Harvest is 3 per cent from air-dried plant material.

The product analysis is performed on the basis of protodioscin. (R. Gyulemetova, M. Tomova, M. Simova, T. Pangarova, Pharmazie, 37, H.4 1982).

RESULTS FROM TESTING OF THE STEROID MIXTURE ACCORDING TO THE INVENTION ON THE IMMUNE SYSTEM OF EXPERIMENTAL ANIMALS

Material and method

Plant material

The substance containing furostanol saponins (FS) was isolated from Tribulus terrestris L (Zigophyllaceae) according to the present invention. Lyophilised FS were stored at 4°C away from light and moisture. The solution used the experiments was prepared ex tempore on the basis of physiologic solution.

Experimental animals and protocol

Male ICR mice (average body weight from 18 to 20 g) were given various oral doses of FS, according to the description shown in Table 1.

Experimental infection

Experimental infection was caused in a mouse by subcutaneous inoculation of 25 to 30 bacterial cells from an 18-hour agar Kl. Pneumonae culture (strain No 52145, Pasteur Institute, Paris). Contamination dose was chosen after running preliminary experiments aimed at obtaining a 50-per cent survival rate. The infection was followed up for eight days, taking account of the subject's general condition, mortality rate (percentage), survival rate and average survival time (AST_8 in days). The protective effect of FS was evaluated along the increase in survival rates and average survival times calculated as differences between experimental groups and controls.

In-vitro antibacterial inhibition test

The sensitivity of Kl. Pneumoniae cell to FS was determined using an adapted version of the Bauer method (4). Petri dishes (100 mm) containing agar were inoculated with 0.2 ml of bacterial suspension (10 cells/ml). Wells (10 mm) were made and filled with 0.2 ml of a FS solution of various concentration. Inhibition areas were measured after incubation for 24 hours at

37°C. Keflin was used as a positive control (Lilly, USA), in minimum inhibition concentrations of 0.150 mg/ml.

Phagocytic and microbicidal activity of alveolar macrophages aMa

Alveolar Ma were obtained through in situ lung lavage by injecting and drawing a 0.1 - 1.0 ml TCM 199 solution containing 20 mM HEPES, 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (Flaw Lab., UK) plus 3 U/ml of heparin (G. Richter, Hungary). The average number of washed aMa from each group was determined and the cells were then placed on incubation plates (BDSL, Scottland) in a concentration of 3 x 10 cells/ml to assess their phagocytic and microbicidal activity using a H^3-thymidine radiometric method.(5)

Results

Anti-infectious protective effect

The results thus obtained show that FS affect the course and outcome of an experimental Kl. Pneumonia-induced infection in mice. In treated animals, mortality rate reached a maximum of 30 to 40 per cent by the 4^{th}-5^{th} day from infection (Figure 1). The peak in mortality rate in the controls was considerably higher (70 to 80 percent) and was observed on the 2^{nd} - 3^{rd} day from infection. To summarise, the administration of FS leads to reduced severity of the clinical picture, lower mortality rate, and prolonged lifetime in treated animals.

The anti-infectious effect of FS varies according to dosage and administration schedules (Table 1). The highest dose administrated (625.0 mg/kg) showed the strongest protective effect in all 10-day administration schedules, starting 35, 25 or 15 day before infection; the peak of the effect was observed where the last FS dose was administered on the 15^{th} day before infection.

In contrast to the marked in-vivo activity, FS do not exhibit in-vitro antibacterial activity. A solution of FS in physiological serum at concentrations of 0.01, 0.025, 0.05, 0.1, 2.0, 5.0, 10.0, 25.0, 50.0, 100.0, 250.0 and 500.0 mg/ml did not suppress Kl. Pneumoniae growth, unlike the marked suppression observed in the **Keflin** group, within a mean area of 21.5 ± 1.0 mm.

Stimulation of aMa

Doses of 625.0 mg/kg cause aMa to increase in number and phagocytic and microbicidal activity with a peak by the 20th day from the last application of FS (Table 2).

Discussion

Unlike other saponins (3), FS do not exert a bactericide effect. FS influence the main effector mechanisms of cell and humoral immunity thus granting a marked protection from infection. The optimum dose of 625.0 mg/kg administered over a sequence of 10 days is very close to the schedule (6) applied in humans. The strongest protective effect obtained with the schedule where the time gap between the last application of FS and infection is 15 days can be explained with the dynamics of aMa activation. The maximum increase in their functional activity is observed by the 20th day which coincides with the acute phase of the infection (day 3 to 5). The great number of aMa with an increased phagocytic and microbicidal capacity prevent aggravation of the infection and reduce mortality rate, thus simultaneously increasing survival rate. Obviously, the protective effect of FS is fully achieved only after a given period of time necessary for the immune system to react. FS activate aMa which play a significant role in the anti-bacterial protection of the lung, in particular in managing infections caused by opportunistic pathogens such as Kl. Pneumonae (6).

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Immunostimulating properties of FS

Table 1: FS-induced anti-infectious effect on experimental infection by Kl. Pneumoniae

	Treatment	Protective effect		
Dose [a]	Treatment [b]	Total dose [c]	Increased survival rate [d]	AST [e]
62.5	15,14,13,12,11,10,9,8,7,6	625.0	32.6	0.8
	25,24,23,24,21,20,19,18,17,16		45.6	1.8
	35,34,33,32,31,30,29,28,27,26		28.5	0.9
	10,9,8,7,6	312.5	10.0	0.4
	20,19,18,17,16		20.0	0.4
	30,29,28,27,26		15.5	0.5
	18,17,16	187.5	42.9	1.2
	7,6	125.0	14.3	3.5
	17,16		33.9	0.4
	27,26		27.3	2.1
50.0	25,23,21,19,17	250.0	16.2	0.4
12.5	25,24,23,22,21,20,19,18,17,16	125.0	10.0	0.4

[a] Single FS dose (mg/kg daily)
[b] Days
[c] Dose (mg/kg)
[d] Change (Δ) in survival rate (%)
[e] Change (Δ) in average survival time (days)

The above results are typical data obtained from three independent experiments using different experimental animals.

Immunomodulating properties of FS

Table 2: Activation of aMa in FS-treated mice

Days of investigation [a]	Number [b]	Phagocytosis [c]	Microbicidal effect [d]
1	202 ± 62 [e]	22.1 ± 2.0 [e]	10.1 ± 1.2
5	213 ± 81 [e]	25.0 ± 3.7	13.3 ± 1.9
10	318 ± 120	29.2 ± 4.4	15.0 ± 2.7
15	323 ± 202	33.0 ± 4.4	16.6 ± 4.8
20	476 ± 88	37.2 ± 1.7	18.0 ± 4.7
30	371 ± 191	32.2 ± 5.8	17.2 ± 2.2
Controls [f]	176 ± 31	21.2 ± 1.9	9.0 ± 1.2

[a] Days from the last application of FS for 10 consecutive days in a doses of 62.5 mg/kg orally

[b] In thousands (x 10^3)

[c] Percentage of aMa-phagocyted bacterial cells

[d] Percentage of bacterial cells killed one hour after phagocytosis by aMa

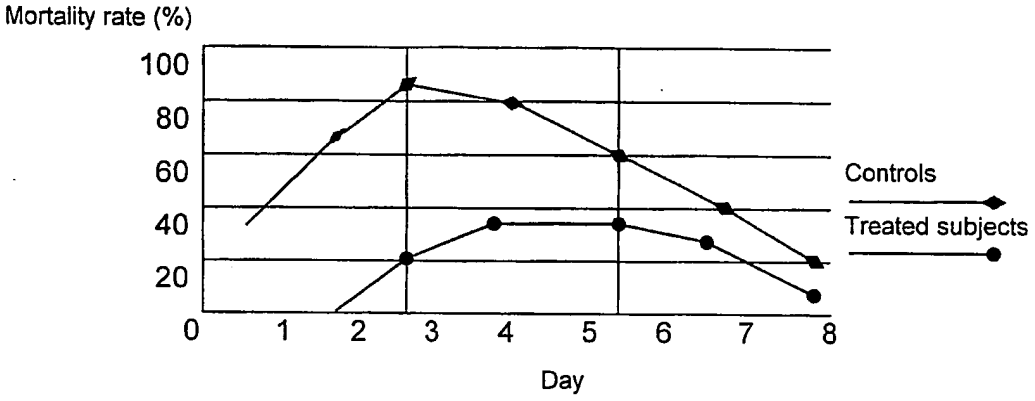
[e] Statistically unreliable ($p > 0.05$ by the Student test)

[f] Controls were fed with physiological serum alone.

The results represent average data from four independent experiments using 10 experimental animals for each group.

Immunomodulating properties of FS

Figure 1. Mortality rates in Kl. Pneumoniae-induced infection in mice



PATENT CLAIMS

1. Standardised steroid saponin mixture containing more than 80 per cent of furostanol saponins.
2. A method to obtain a standardised steroid saponin mixture as the one described in Claim 1, from *Tribulus terrestris* by the means of extraction with water-saturated n-butanol, concentration and drying, characterised in that the first extraction being performed using aqueous methanol in a 1:1 water / methanol ratio at a temperature of 52 to 55°C, followed by purification with active carbon and filtering; after concentration and full removal of the methanol and subsequent extraction with methylene chloride, the purified water extract is acidified up to pH 3 - 5 using hydrochloric acid, then, after extraction with n-butanol and concentration it is proceeded either to: dropping into acetone, leaving for about 24 hours at a temperature of 10 to 15°C, filtering, acetone washing, and drying or to: washing the concentrate with 0.5 - 1.5-per cent sodium hydroxide solution and, after separating the two layers, washing the butanol layer with water up to pH 7, vacuum-concentrating the butanol extracts until the organic solvent is fully removed, and drying the water solution.
3. Application of the standardised mixture described in Claim 1 in the synthesis of drugs with immunomodulating and/or immunostimulating effect.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BG 03/00043

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K35/78 A61P37/02 A61P37/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YAN W ET AL: "Steroidal saponins from fruits of Tribulus terrestris" PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 45, no. 4, June 1997 (1997-06), pages 811-817, XP004293208 ISSN: 0031-9422 the whole document ---	1
X	GJULEMETOWA R ET AL: "UEBER DIE BESTIMMUNG VON FUROSTANOLSAPONINEN IM PRAEPARAT TRIBESTAN" PHARMAZIE, VEB VERLAG VOLK UND GESUNDHEIT. BERLIN, DD, vol. 37, no. 4, 1982, page 296 XP001154500 ISSN: 0031-7144 the whole document	1
Y	--- -/--	2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 May 2004	17/06/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Thalmair, M

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Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ADAIKAN P G ET AL: "HISTORY OF HERBAL MEDICINES WITH AN INSIGHT ON THE PHARMACOLOGICAL PROPERTIES OF TRIBULUS TERRESTRIS" AGING MALE, PARTHENON, LONDON, GB, vol. 4, no. 3, 2001, pages 163-169, XP009015949 ISSN: 1368-5538	1
Y	page 166, left-hand column, paragraph 2	2
Y	ACHENBACH H ET AL: "CARDIOACTIVE STEROID SAPONINS AND OTHER CONSTITUENTS FROM THE AERIAL PARTS OF TRIBULUS CISTOIDES" PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 35, no. 6, 1 April 1994 (1994-04-01), pages 1527-1543, XP000576854 ISSN: 0031-9422 page 1529; table 1	2
X	PETIT PIERRE R ET AL: "Steroid saponins from fenugreek seeds: Extraction, purification, and pharmacological investigation on feeding behavior and plasma cholesterol" STEROIDS, vol. 60, no. 10, 1995, pages 674-680, XP000876727 ISSN: 0039-128X the whole document	1
X	MURAKAMI TOSHIYUKI ET AL: "Medicinal foodstuffs. XVII. Fenugreek seed. (3): Structures of new furostanol-type steroid saponins, trigoneosides Xa, Xb, XIb, XIIa, XIIb, and XIIIa, from the seeds of Egyptian *Trigonella foenum-graecum* L" CHEMICAL AND PHARMACEUTICAL BULLETIN (TOKYO), vol. 48, no. 7, July 2000 (2000-07), pages 994-1000, XP000180963 ISSN: 0009-2363 page 994, left-hand column, line 5	1,3
A	US 2002/082780 A1 (ALEXIS BRIAN) 27 June 2002 (2002-06-27) the whole document	1-3

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BG 03/00043

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims 1 and 3 concern any standardised steroid saponin mixture containing more than 80 per cent of furostanol saponins, whereas the description provides support and disclosure only for such a steroid saponin mixture obtained from the epigeous part of Tribulus terrestris (Article 84 and/or 83 EPC).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/BG 03/00043

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002082780 A1	27-06-2002	US 6343258 B1	29-01-2002
		US 2003211185 A1	13-11-2003
		BG 106408 A	31-10-2002
		EP 1202630 A1	08-05-2002
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Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *of inventorship (Rule 4.17(iv)) for US only*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: STANDARDISED STEROID SAPONIN MIXTURE, A METHOD OF ITS OBTAINING AND APPLICATION

(57) Abstract: The invention relates to a standardised steroid saponin mixture obtained from the epigeous part of Tribulus terrestris L., containing more than 80 per cent of furostanol saponins, a method of obtainment and its application as an immunostimulator and immunomodulator.

WO 2004/064852 A1

AMENDED CLAIMS
[Received by the International Bureau on 22 July 2004 (22.07.04); claims 1 to 3 have been replaced by amended claims 1 to 3;]

+ Statement

PATENT CLAIMS

1. Standardised steroid saponin mixture, obtained from the epigeous part of *Tribulus terrestris L.*, containing more than 80 per cent of furostanol saponins.
2. A method to obtain a standardised steroid saponin mixture as the one described in Claim 1, from *Tribulus terrestris* by the means of extraction with water-saturated n-butanol, concentration and drying, characterised in that the first extraction being performed using aqueous methanol in a 1:1 water / methanol ratio at a temperature of 52 to 55°C, followed by purification with active carbon and filtering; after concentration and full removal of the methanol and subsequent extraction with methylene chloride, the purified water extract is acidified up to pH 3 - 5 using hydrochloric acid, then, after extraction with n-butanol and concentration it is proceeded either to: dropping into acetone, leaving for about 24 hours at a temperature of 10 to 15°C, filtering, acetone washing, and drying or to: washing the concentrate with 0.5 - 1.5-per cent sodium hydroxide solution and, after separating the two layers, washing the butanol layer with water up to pH 7, vacuum-concentrating the butanol extracts until the organic solvent is fully removed, and drying the water solution.
3. Application of the standardised mixture described in Claim 1 in the synthesis of drugs with immunomodulating and/or immunostimulating effect.

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Applicant's file reference: SOFARMA 3-43

Sofia, July, 16, 2004

STATEMENT UNDER ARTICLE 19 (1)

Re: International Patent Application № PCT/ BG / 03 / 00043 with applicant SOPHARMA AD

In the response to the PCT Notification of transmittal of the international search report or the declaration pursuant to PCT Rule 44.1 /Regulations under PCT/ of 17 June 2004.
Considering the received Search Report , please note the applicant is making an amendment at the first claim as he restrict the text in claim 1 to the disclosure in the description, namely "Standardised steroid saponin mixture, ***obtained from the epigeous part of Tribulus terrestris .L,*** containing more than 80 per cent of furostanol saponins."

Thereby the formed claim distinguish the invention according to the present application from the cited resources in the Search Report – PETIT PIERRE R ET AL: "*Steroid saponins from fenugreek seeds: Extraction, purification and pharmacological investigation on feeding behavior and plasma cholesterol*" and MURAKAMI TOSHIYUKI ET AL: "*Medicinal foodstuffs. XVII. Fenugreek seed. Structures of new furostanol-type steroid saponins, trigoneosides Xa, Xb, XIb, XIIa, XIIb, and XIIIa, from the seeds of Egyptian Trigonella foenum-graecum L*" and ACHENBACH H ET AL: "*Cardioactive steroid saponins and other constituents from the aerial parts of tribulus cistoides*".

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Account: 116-027-141-8=USD, Account: 106-027-142-0=BGL, Account: 146-027-140-3=EURO